Noble, R. B. (1968) *J. Lipid Res. 9*, 693–700.
Phillips, M. C. (1972) *Prog. Surf. Membr. Sci. 5*, 139–214.
Phillips, M. C., & Chapman, D. (1968) *Biochim. Biophys. Acta 163*, 301–313.
Phillips, M. C., & Krebs, K. E. (1986) *Methods Enzymol. 128*, 387–403.
Phillips, M. C., Evans, M. T. A., & Hauser, H. (1975a) *Adv. Chem. 144*, 217–230.
Phillips, M. C., Graham, D. E., & Hauser, H. (1975b) *Nature (London) 254*, 154–155.
Phillips, M. C., Hauser, H., Leslie, R. B., & Oldani, D. (1975c) *Biochim. Biophys. Acta 406*, 402–414.
Pownall, H. J., Massey, J. B., Kusserow, S. K., & Gotto, A. M. (1979) *Biochemistry 18*, 574–579.
Reijngoud, D.-J., & Phillips, M. C. (1982) *Biochemistry 21*, 2969–2975.
Scanu, A. M. (1979) in *The Biochemistry of Atherosclerosis* (Scanu, A. M., Ed.) pp 3–8, Marcel Dekker, New York.
Scanu, A. M., & Edelstein, C. (1971) *Anal. Biochem. 44*, 576–588.
Scanu, A. M., Byrne, R. E., & Mihovilovic, M. (1982) *CRC Crit. Rev. Biochem. 13*, 109–140.
Schaefer, E. J., Eisenberg, S., & Levy, R. I. (1978) *J. Lipid Res. 19*, 667–687.
Segrest, J. P., Jackson, R. L., & Gotto, A. M., Jr. (1974) *FEBS Lett. 38*, 247–253.
Skipski, V. P. (1972) in *Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism* (Nelson, G. J., Ed.) pp 471–583, Wiley-Interscience, New York.
Sokoloff, L., & Rothblat, G. H. (1974) *Proc. Soc. Exp. Biol. Med. 146*, 1166–1172.
Tall, A. R., & Lange, Y. (1978a) *Biochim. Biophys. Acta 513*, 185–195.
Tall, A. R., & Lange, Y. (1978b) *Biochem. Biophys. Res. Commun. 80*, 206–212.
Tall, A. R., & Small, D. M. (1980) *Adv. Lipid Res. 17*, 1–51.

Laser Flash Photolysis as a Probe of Redox Protein–Membrane Interactions: Effect of Binding of Spinach Plastocyanin and Horse Cytochrome *c* to Lipid Bilayer Vesicles on the Kinetics of Reduction by Flavin Semiquinone†

Velu Senthilathipan and Gordon Tollin*
Department of Biochemistry, University of Arizona, Tucson, Arizona 85721
Received June 23, 1988; Revised Manuscript Received September 16, 1988

ABSTRACT: Spinach plastocyanin binds to both electrically neutral and positively charged lipid bilayer vesicles, whereas cytochrome *c* only binds electrostatically to negatively charged vesicles. Laser flash photolysis using lumiflavin semiquinone as a reductant demonstrates that the reactivity of plastocyanin is increased as much as 6-fold when it is membrane bound whereas the rate constant for cytochrome *c* reduction is decreased by approximately a factor of 3. Membrane-bound plastocyanin reduction occurs via a two-step mechanism, probably involving prior association of lumiflavin semiquinone with the bilayer. In contrast, cytochrome *c* reduction in the membrane-bound state follows simple second-order kinetics, implying that the redox site in the bound state is still accessible to lumiflavin semiquinone in solution, although the rate constant is decreased by approximately 3-fold. These results are interpreted as indicating that the bilayer–protein interaction with plastocyanin leads to a steric blockage of the electron-transfer site from the aqueous phase. Little or no hindrance of the redox site occurs with cytochrome *c*, suggesting a high degree of mobility of this protein on the bilayer surface. Although the increase in plastocyanin reactivity upon binding to the bilayer is quite interesting, its cause remains unclear and requires further study. The results illustrate the utility of laser flash photolysis as a probe of membrane–protein interactions.

It is well-known that many physiological electron-transfer systems are membrane bound and that such membrane binding and organization facilitates compartmentalization and directional electron transfer. Mitochondrial cytochrome *c*, which functions in respiratory electron transport, has been shown to bind electrostatically to vesicles formed from mixtures of negatively charged and neutral phospholipids, and this interaction has been studied quite extensively (Birrel & Griffith, 1976; Brown & Wüthrich, 1977a,b; Chapman & Urbina, 1971; Green & Fleischer, 1963; Kimelberg & Lee, 1969; Kimelberg & Paphadjopoulos, 1971; Mustonen et al., 1987; Nicholls & Malviya, 1973; Nicholls, 1974; Overfield & Wraight, 1980a,b; Quinn & Dawson, 1969; Senthilathipan & Tollin, 1986; Steinemann & Laugher, 1971; Van & Griffith, 1975; Vanderkooi et al., 1973a,b). Recent work (Gupte & Hackenbrock, 1988a,b) has provided evidence that, under physiological conditions, the rate of diffusion of cytochrome *c* along the surface of the inner mitochondrial membrane may be the rate-limiting step in the transport of electrons between the cytochrome bc_1 and cytochrome oxidase complexes.

Another electron-transfer protein, plastocyanin, is located on the inside of the photosynthetic thylakoid membrane (Haehnel et al., 1981; Hauska et al., 1971) and also acts as a mobile electron carrier (Olsen, 1982; Takanao et al., 1982) shuttling electrons between the cytochrome b_6/f complex and photosystem I. Unlike cytochrome *c*, there are relatively few reports in the literature on model studies of plastocyanin–

† This work was supported in part by the Division of Chemical Sciences, Office of Basic Energy Sciences, Office of Energy Research, U.S. Department of Energy.
* To whom correspondence should be addressed.

0006-2960/89/0428-1133$01.50/0

membrane interactions (Botin & Mathis, 1985; Farver & Pecht, 1981; Farver et al., 1982; Senthilathipan & Tollin, 1987; Rich et al., 1987).

From the X-ray crystallographic structure of tuna cytochrome *c* (Swanson et al., 1977), which is very similar to horse cytochrome *c*, it is clear that the heme prosthetic group is only partially exposed in a hydrophobic cleft and is surrounded by a cluster of positively charged amino acid side chains. A positive electrostatic potential completely covers the exposed heme edge (Weber & Tollin, 1985). Various studies have shown that electron transfer to cytochrome *c* most probably takes place at, or close to, the exposed heme edge (Cusanovich & Miller, 1974; Koppenol & Margoliash, 1982; Staudenmayer et al., 1976, 1977; Tollin et al., 1986). Inasmuch as the surface of the heme group which is accessible to water molecules forms only 0.6% of the total surface area of the protein (Stellwagen, 1978), questions relating to the orientation of this region relative to the membrane, and its accessibility to molecules located in the membrane and in the aqueous phase, are significant in terms of biological function.

Cytochrome *c* binds strongly to negatively charged membranes, and there is evidence which suggests that the heme region penetrates hydrophobically into the bilayer (Brown & Wüthrich, 1977a; Nicholls & Malviya, 1973; Nicholls, 1974; Overfield & Wraight, 1980b; Senthilathipan & Tollin, 1986; Gulik-Krzywick et al., 1969; Papahadjopoulos et al., 1975; Szebeni & Tollin, 1988). However, Brown and Wüthrich (1977a,b) have concluded on the basis of ^{13}C and ^{1}H NMR and spin-label studies of the binding of cytochrome *c* to 1:4 cardiolipin–phosphatidylcholine vesicles that the heme edge was pointed away from the negatively charged membrane. This is an unexpected orientation in view of the charge distribution on the cytochrome surface. Furthermore, Van and Griffith (1975) found that cytochrome *c* bound to negatively charged membranes had little or no effect on the fluidity gradient of spin-labels attached to the fatty acid side chains. These somewhat conflicting observations suggest that there may be a high degree of mobility of the cytochrome *c* molecule on the vesicle surface, involving both translational movement and perhaps even some degree of rotational motion, as well as a highly dynamic situation as regards cytochrome penetration into the membrane interior. As will be discussed below, the present studies support this point of view.

Relatively few investigations have been performed which address the question of the accessibility of the heme prosthetic group of membrane-bound cytochrome *c* to reductants located in the aqueous phase. Nicholls and Malviya (1973) have observed that binding of cytochrome *c* to negatively charged liposomes decreases the second-order rate constant for ascorbate reduction by more than a factor of 50, and Wainwright et al. (1978) found that reduction of cytochrome *c* by the hydrated electron was completely blocked upon binding to negatively charged vesicles. Taken at face value, these results imply a significant steric hindrance of the heme edge caused by association of cytochrome *c* and negative bilayers. However, these reactions involve charged reductants and were only investigated at a single ionic strength. Thus, electrostatic effects could have occurred which, unless assessed via ionic strength dependence studies, could have influenced reaction rates and hence complicated analysis of steric effects.

The shape of the plastocyanin molecule (Colman et al., 1978) resembles a slightly flattened cylinder with the copper atom located at one end of the protein. Previous studies have suggested that two regions on the plastocyanin surface, one hydrophobic and one negatively charged, function as interaction sites for electron-transfer reactions (Farver & Pecht, 1981; Farver et al., 1982; Colman et al., 1978; Lappin et al., 1979; Cookson et al., 1980; Freeman, 1981; Guss & Freeman, 1983; Takabe et al., 1980, 1984). Thus, the structure/reactivity situation for this protein is rather different from that of cytochrome *c*. The hydrophobic site involves the exposed edge of the His-87 imidazole ring, which is a ligand for the copper atom and which is almost precisely level with the northern molecular boundary. The other interaction site is in the region of Tyr-83 and the negatively charged patch proximal to it. To our knowledge, no investigations of the effect of binding of plastocyanin to bilayers on the kinetics of copper reduction have been reported.

In previous work from this laboratory, we have shown that both cytochrome *c* and plastocyanin can accept electrons from the membrane-bound chlorophyll triplet state, when these redox proteins are electrostatically bound to the surface of negatively and positively charged bilayers, respectively (Senthilathipan & Tollin, 1986, 1987). In order to explain the large degree of triplet quenching by bound protein which occurred in these experiments, we postulated that cytochrome *c* and plastocyanin penetrated to some extent into the membrane interior. This hypothesis has received further experimental support by studies using photoaffinity labeling (Szebeni & Tollin, 1988). In the present experiments, we have compared the kinetics of reduction of free and membrane-bound cytochrome *c* and plastocyanin by exogenous free flavin neutral semiquinone radicals (generated in the outer aqueous phase by laser flash photolysis) as a means of probing the accessibility and reactivity of the redox centers, and have correlated the results with the nature of the interaction between the bilayer and the redox protein. In this case, the reductant is electrically uncharged [cf. Tollin et al. (1986)], and hence no complications from electrostatic effects should occur, although, as will be documented below, some kinetic complexities can arise due to the association of the reductant with the bilayer. These are easily recognized, however, from nonlinear concentration effects. The results illustrate the utility of this approach for obtaining insights into redox protein–membrane interactions.

Materials and Methods

Horse heart cytochrome *c* (oxidized) (type IV) was obtained from Sigma Chemical Co. and used without further purification. Plastocyanin from spinach was isolated according to Yocum (1982). The positively charged surfactant dioctadecyldimethylammonium bromide ($DODAC^+$)[1] was purchased from Eastman Kodak. The corresponding chloride salt was obtained by passing a solution of the bromide 2 or 3 times through Amberlite IRA-410 C.P. ion-exchange resin, with subsequent evaporation of the eluate. The potassium salt of the negative surfactant dihexadecyl phosphate (DHP^-) was produced from the corresponding free acid (Sigma Chemical Co.). Preparation of lumiflavin was as previously described (Simondsen & Tollin, 1983). All other materials were also as previously described (Senthilathipan & Tollin, 1985; Fang & Tollin, 1983).

Bilayer vesicles of the small unilamellar type were prepared by the injection method (Batzri & Korn, 1973), as described in Fang and Tollin (1983). Varying amount of DHP^- were incorporated into egg PC bilayers to produce negatively charged vesicles, and $DODAC^+$ was similarly used to form

[1] Abbreviations: DHP^-, dihexadecyl phosphate; $DODAC^+$, dioctadecyldimethylammonium chloride; egg PC, egg phosphatidylcholine; LF, lumiflavin; $LFH^\cdot$, lumiflavin semiquinone; EDTA, disodium salt of ethylenediaminetetraacetic acid.

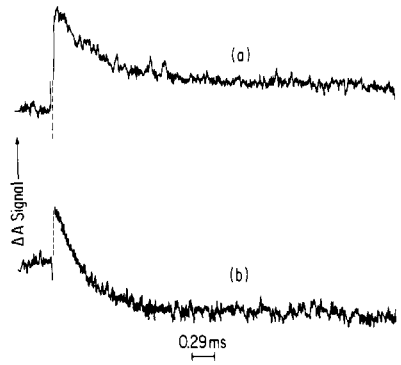

FIGURE 1: Transients obtained at 580 nm when 5 mM phosphate buffer containing lumiflavin (60 μM) and EDTA (1 mM) (pH 7.0) was illuminated with a laser flash. (Upper curve) Decay curve of lumiflavin semiquinone radicals in the absence of any redox proteins, due to disproportionation. (Lower curve) Decay curve of lumiflavin semiquinone radicals in the presence of spinach plastocyanin (10 μM). Curve goes below preflash base line due to plastocyanin reduction.

positively charged membranes [cf. Fang and Tollin (1983)]. The lipid concentration (the total concentration when mixed lipids were used) was 2.2 mM. Either phosphate buffer (5–40 mM, pH 7.0) or 50 mM betaine (pH 6.5), containing lumiflavin (60 μM) and EDTA (1 mM), was used as the suspending medium. The EDTA served as a reductant for the lumiflavin triplet state generated by the laser flash to produce the lumiflavin semiquinone [cf. Tollin et al. (1986)]. The zwitterionic betaine was used to provide a low ionic strength solvent with better osmotic characteristics than deionized water (Senthilathipan & Tollin, 1985). Plastocyanin was added to the vesicle suspension to its final concentration (≤15 μM) from a 1.5 mM stock solution, and cytochrome *c* was added to the vesicle suspension to its final concentration (≤50 μM) from a 3.2 mM stock solution. Thus, these proteins were only present in the external aqueous volume of the vesicle suspension.

The laser flash apparatus was similar to that described previously (Tollin et al., 1979; Hurley et al., 1980, 1981). It utilized a 10-ns-pulsed N_2 laser (Molectron UV 22) which pumped a dye laser consisting of a 10 mM solution in ethanol of the dye C-450 obtained from Photochemical Research Associates, Inc. The excitation wavelength was 450 nm. Decay curves following a laser flash were obtained by using a Biomation Model 8100 waveform recorder and a Nicolet 1070 signal averager, with four to eight decays averaged for each data set. All measurements were carried out under anaerobic conditions at room temperature (22 ± 1 °C). Protein reduction by lumiflavin semiquinone was generally monitored by absorbance changes at 575–580 nm, where both the semiquinone and the protein absorption contributed. The methodology and rationale for using laser photolytic generation of flavin semiquinones to study redox protein electron-transfer mechanisms are described in Tollin et al. (1986). Under the experimental conditions used, the redox protein concentration was always 1–2 orders of magnitude larger than the flavin semiquinone concentration generated by the laser pulse, and thus protein reduction followed pseudo-first-order kinetics.

Results

Figure 1 shows typical flash-induced transient decays measured at 580 nm during a 5-ms time interval with and without added plastocyanin. The upper curve corresponds to the second-order disproportionation of lumiflavin semiquinone to form the oxidized and fully reduced flavin [cf. Tollin et al. (1986)]. The pseudo-first-order decay below the preflash base line in the lower curve is due to plastocyanin reduction by the semiquinone, which is competitive with the disproportionation reaction. Semilog plots of such decay curves at various added protein concentrations were used to obtain k_{obsd} values for protein reduction.

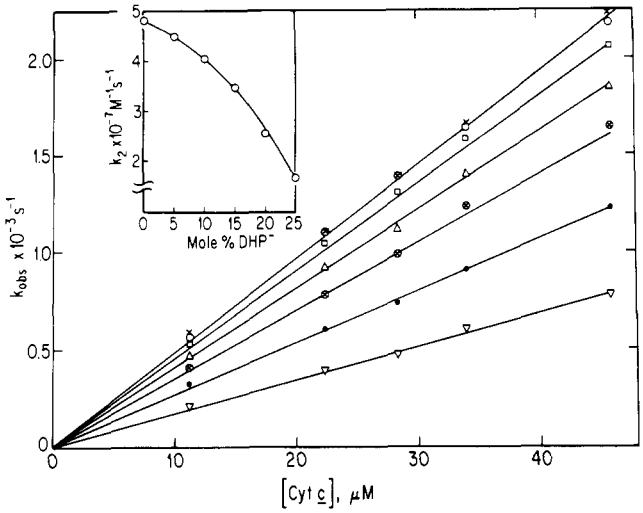

FIGURE 2: Kinetics of reduction of horse heart cytochrome *c* by lumiflavin semiquinone radicals when 100% egg PC and 5–25% DHP^-–egg PC liposomes were suspended in 5 mM phosphate buffer (pH 7.0). (×) No vesicles; (○) 100% egg PC; (□) 5% DHP^-; (△) 10% DHP^-; (⊗) 15% DHP^-; (●) 20% DHP^-; (▽) 25% DHP^-. Inset: Effect of mole percent DHP^- incorporated on the second-order rate constant for the reduction of cytochrome *c* by lumiflavin semiquinone radicals.

Table I: Plastocyanin Reduction by Flavin Semiquinone Radicals

system	k_2 (×10^{-7} M^{-1} s^{-1})	k_1 (s^{-1})
phosphate buffer only (no liposomes)	4.2	
neutral liposomes (100% PC)	13	400
5% $DODAC^+$, 95% PC	15	500
10% $DODAC^+$, 90% PC	17	625
15% $DODAC^+$, 85% PC	18.5	790
20% $DODAC^+$, 80% PC	21	920
25% $DODAC^+$, 75% PC	25	1000

Figure 2 shows the effect of increasing the mole percent of DHP^- incorporated into the vesicle on the observed pseudo-first-order rate constants for reduction of varying concentrations of cytochrome *c* by flavin semiquinone radicals, when the vesicles were suspended in 10 mM phosphate buffer (pH 7.0). Cytochrome *c* was added up to a final concentration of 50 μM; addition of larger amounts caused aggregation of the vesicles. The calculated second-order rate constant for the reduction of cytochrome *c* was found to be the same in the presence of neutral liposomes and in phosphate buffer with no liposomes (Figure 2, Table I) and is comparable to that obtained previously (Meyer et al., 1983) under somewhat different experimental conditions. The lack of effect of uncharged vesicles is consistent with previous studies which show that cytochrome *c* does not bind to electrically neutral bilayers (Green & Fleischer, 1963; Vanderkooi et al., 1973a; Nicholls & Malviya, 1973). Reduction of cytochrome *c* in the presence of negatively charged vesicles followed second-order kinetics over the concentration range used. The calculated second-order rate constants were inversely proportional to the mole percent of DHP^- incorporated (cf. Figure 2) and varied over a 2.8-fold range.

In Figure 3 is shown the effect of the presence of neutral vesicles and of $DODAC^+$ incorporation into vesicles on the observed rate constant for reduction of varying concentrations of plastocyanin by flavin semiquinone radicals in 5 mM

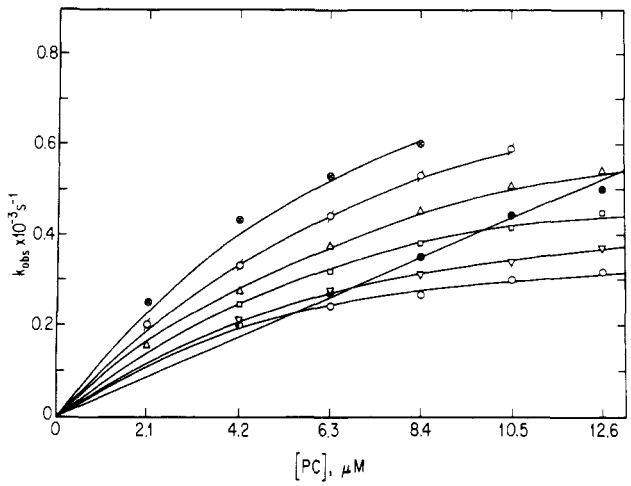

FIGURE 3: Kinetics of reduction of spinach plastocyanin by flavin semiquinone radicals when 100% egg PC and 5–25% $DODAC^+$–egg PC liposomes were suspended in 5 mM phosphate buffer. Solid curves for vesicle data are based on theoretical fits to the data points obtained by nonlinear least-squares analysis using a two-step mechanism (see text). (●) No vesicles; (○) 100% egg PC; (▽) 5% $DODAC^+$; (□) 10% $DODAC^+$; (△) 15% $DODAC^+$; (∅) 20% $DODAC^+$; (⊗) 25% $DODAC^+$.

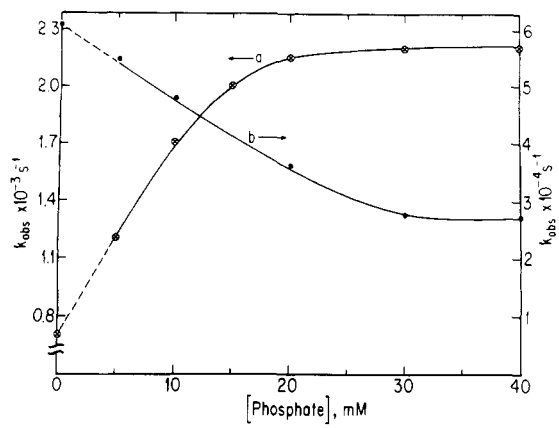

FIGURE 4: Kinetics of reduction of redox proteins as a function of buffer concentration. (a) Observed rate constants for horse heart cytochrome *c* reduction by flavin semiquinone radicals when 15% DHP–85% egg PC liposomes were suspended in various concentrations of phosphate buffer (pH 7.0). Cytochrome concentration was 45 μM. (b) Observed rate constants for spinach plastocyanin reduction by flavin semiquinone radicals when 25% $DODAC^+$–75% egg PC liposomes were suspended in various concentrations of phosphate buffer (pH 7.0). Plastocyanin concentration was 8.0 μM. The observed rate constants obtained in 50 mM betaine (pH 6.5) were used as the points for zero buffer concentration.

phosphate buffer (pH 7.0). Plastocyanin was added to a final concentration of 15 μM; further addition caused vesicle aggregation in positively charged liposomes. In the case of plastocyanin, although second-order kinetics were obtained in the absence of vesicles,[2] nonlinear concentration dependencies were observed for all of the vesicle systems, including neutral vesicles. This indicates the occurrence of a reaction mechanism which involves a minimum of two successive steps, one of which is second order and the other of which is first order. This will be discussed further below. Table I lists values for the second-order and limiting first-order rate constants obtained by a nonlinear least-squares fit to the data for a two-step mechanism of the above type [cf. Simondsen and Tollin (1983)]. It is clear from these results that the rate of reduction of plastocyanin increased upon binding to neutral vesicles and became still faster with an increase in the mole percent of $DODAC^+$ incorporated, in sharp contrast to the results obtained with cytochrome *c*. We have also found that suspending positively charged vesicles (25 mol % $DODAC^+$) in 50 mM betaine slightly accentuated the enhancement in reduction kinetics observed in 10 mM phosphate (data not shown). This would be expected for an effect which is mediated by electrostatic interactions.

Figure 4 demonstrates the effects of the buffer concentration on the reduction of cytochrome *c* and plastocyanin in negatively and positively charged liposomes, respectively. Complex formation between the redox proteins and the charged vesicles is facilitated by low buffer concentrations (i.e., low ionic strength) (Senthilathipan & Tollin, 1986, 1987). The rate constant for reduction of cytochrome *c* increased with the buffer concentration to a limiting value. It should be noted that 20 mM phosphate buffer was able to completely screen the electrostatic effects of 15 mol % DHP^- and that under these conditions the system behaved like 100% PC liposomes. Unlike cytochrome *c*, the rate of reduction of plastocyanin decreased with the buffer concentration, again reaching a limiting value. As would be expected, the higher mole percent of surfactant in this case required a higher buffer concentration to cancel the electrostatic interaction. The "zero buffer concentration" points, which were obtained in 50 mM betaine, are consistent with the data obtained in phosphate buffer. This indicates that the buffer ions were not exerting any specific effects.

Discussion

Liposomes are quasi-spherical multimolecular assemblies in which the lipid bilayer separates an inner aqueous compartment from the bulk water phase. The polar head groups of the lipids are exposed to the aqueous phase on both sides of the bilayer, while the hydrocarbon chains of the lipids dissolve within themselves, comprising the inner core of the bilayer. Charged surfactants will orient themselves within the bilayer so that they contribute a net electrostatic potential to the inner and outer membrane surfaces. Protein molecules can thus associate both electrostatically and hydrophobically with these bilayer vesicles.

The following mechanism can explain the kinetics of flash-induced reduction of plastocyanin and cytochrome *c* by flavin semiquinone radicals [cf. Tollin et al. (1986)]. In these

$$LF(ox) + e^- + H^+ \xrightarrow{h\nu} LFH^\cdot \quad (1)$$

$$2LFH^\cdot \rightarrow LF(ox) + LF(red) \quad (2)$$

$$P(ox) \rightarrow P(ox)_m \quad (3)$$

$$P(ox) + LFH^\cdot \rightarrow P(red) + LF(ox) \quad (4)$$

$$P(ox)_m + LFH^\cdot \rightarrow P(red)_m + LF(ox) \quad (5)$$

equations, P(ox), P(red), and the subscript m stand for oxidized, reduced, and membrane-bound protein, respectively, and LF(ox), LF(red), and $LFH^\cdot$ refer to oxidized lumiflavin, fully reduced lumiflavin, and the neutral lumiflavin semiquinone radical, respectively. In the presence of light and EDTA (reaction 1), lumiflavin forms semiquinone radicals via triplet-state quenching. This occurs predominantly in the aqueous phase, due to the solubility properties of EDTA. Lumiflavin semiquinone either can disproportionate to form oxidized and reduced lumiflavin (reaction 2) in the absence of electron acceptors or can reduce redox proteins, either free or membrane bound (reactions 4 and 5). The latter equations assume

[2] The calculated second-order rate constant is significantly larger than that obtained previously [4.8 × 10^7 M^{-1} s^{-1} vs 2.4 × 10^7 M^{-1} s^{-1}; cf. Tollin et al. (1986)]. This may be due to the differences in reaction conditions, although this was not explicitly investigated.

that protein reduction occurs via $LFH^{\cdot}$ located in the aqueous phase. However, as we shall see below, this need not necessarily be the case. Free oxidized protein is in equilibrium with its membrane-bound counterpart (reaction 3). In the case of charged vesicles, the extent of the forward direction of reaction 3, i.e., electrostatic complex formation between the redox protein and the charged membrane, will be directly proportional to the amount of surface charge on the vesicles, and inversely proportional to the ionic strength of the medium. Conversely, electrostatic complex dissociation should be facilitated by high ionic strength and low surface charge. All other things being equal, one would predict that the rate of protein reduction in the aqueous phase would be faster than for the membrane-bound protein, due to slower diffusion of the macromolecule–vesicle complex and to possible steric constraints on the access of the exogenous reductant to the protein redox center in the bound protein.

In the present studies, the rate of reduction of cytochrome *c* by flavin semiquinone radicals (Figure 2) was found to be the same in 10 mM phosphate buffer whether or not neutral liposomes were present. This is consistent with the absence of stable complex formation between the electrically neutral membrane surface and cytochrome *c*. As the mole percent of DHP^{-} increased in the membrane, evidently an increasing amount of cytochrome *c* was bound (Senthilathipan & Tollin, 1986) and the observed rate of reduction decreased. In all cases, second-order kinetics were obeyed, which is consistent with $LFH^{\cdot}$ in the aqueous phase reacting in a bimolecular manner with the cytochrome *c*, both free and membrane bound. The rate constants for these two reactions were, under all experimental conditions, apparently close enough to one another that it was not possible to resolve the individual reactions; i.e., the decay curves always appeared to be single exponential. When 25 mol % DHP^{-} was incorporated, the observed rate constant for cytochrome *c* reduction was 2.8 times smaller than in phosphate buffer with neutral or no liposomes. This is a relatively small decrease in rate constant, which could be due mainly to diffusional effects. This result implies that there was little or no steric blockage of the heme site upon binding to the vesicles. We infer, therefore, that the orientation of the cytochrome *c* molecule on the membrane surface is highly dynamic and that penetration of lumiflavin semiquinone from the aqueous phase to the exposed heme edge occurred with essentially no restriction other than that due to diffusion. Calculations of the positive electrostatic potential on the surface of tuna cytochrome *c* (Weber & Tollin, 1985), which is highly homologous with horse cytochrome *c*, indicate that the coverage of the front surface by the potential is extensive enough that the cytochrome molecule might be able to rotate by as much as 90° without much diminution of the electrostatic interaction with the bilayer. This is a quite different point of view from that which was obtained on the basis of the kinetic measurements using ascorbate and the hydrated electron (see above). Another factor in addition to steric and diffusional constraints that might have been involved in the observed decrease in reduction rate constant is a decrease in the cytochrome *c* redox potential upon binding to the vesicles. However, this is likely to be no more than an approximately 30-mV change (Nicholls, 1974), which based upon previous results with lumiflavin semiquinone (Tollin et al., 1986) would be expected to cause a rate constant decrease of only 10–20%.

When 100% egg PC liposomes were suspended in 5 mM phosphate buffer, the rate of reduction of plastocyanin was increased slightly relative to that in buffer with no liposomes (Figure 3, Table I). Furthermore, the concentration dependence became nonlinear. Thus, unlike cytochrome *c*, an interaction between plastocyanin and the uncharged vesicles was clearly occurring, presumably via hydrophobic interactions.

When $DODAC^{+}$ was incorporated into the neutral vesicles, the rate of reduction of plastocyanin increased still further and continued to become larger as the mole percent increased (Figure 3). Again, nonlinear concentration dependencies were obtained. As was the case with cytochrome *c*, it was not possible to resolve the individual kinetic contributions of the bound and unbound proteins. With 25 mol % $DODAC^{+}$ incorporated, the calculated second-order rate constant for reduction of plastocyanin obtained from the fit to the data was approximately 6 times that in 5 mM phosphate buffer with no liposomes. The simplest interpretation of the nonlinear concentration effects is that the mechanism involved a prior association of $LFH^{\cdot}$ with the bilayer, followed by intravesicle electron transfer to the bound plastocyanin. This change in the mechanism of plastocyanin reduction from simple second order, as observed with cytochrome *c*, to a multistep process implies that the direct access of $LFH^{\cdot}$ molecules in the aqueous phase to the plastocyanin electron-transfer site was highly restricted upon binding of the plastocyanin to the vesicle, both for neutral and for positively charged membranes, and that reduction occurred from within the bilayer rather than from the aqueous phase. This implies that plastocyanin reduction by lumiflavin semiquinone occurred predominantly at the "hydrophobic site" and that this region of the molecule was highly buried within the vesicle for both neutral and positively charged bilayers.

Both the second-order and limiting first-order rate constants were observed to increase with increasing positive charge on the bilayer. A possible explanation for this behavior is an expansion of the bilayer surface structure caused by electrostatic repulsion between the positively charged surfactant head groups, which allowed easier penetration of the bilayer surface by $LFH^{\cdot}$ and more facile diffusion within the bilayer to the bound plastocyanin. The basis for the increased reactivity of plastocyanin toward $LFH^{\cdot}$ when bound to the membrane, as compared to free in solution, is not clear at present. Several possibilities exist, e.g., structural changes in the protein, redox potential changes, etc. This very interesting observation requires further study. However, it is reminiscent of the report by Vanderkooi (1976) that the steady-state photoreduction of cytochrome *c* by electronically excited phenothiazine occurred much more rapidly when the cytochrome was bound to sonicated dispersions of lecithin and cardiolipin than when it was free in solution. In this case, however, it was presumed that the reductant was partitioned predominantly into the phospholipid phase and that the reaction occurred from within the bilayer.

The results presented here demonstrate the usefulness of laser flash photolysis measurements of the kinetics of reduction by flavin semiquinones in providing both structural and reactivity information concerning membrane-bound redox proteins. We anticipate that this approach should prove to be generally applicable as a probe of membrane–protein interactions.

Registry No. $LFH^{\cdot}$, 34533-61-4; DHP, 2197-63-9; $DODAC^{+}\cdot Cl^{-}$, 107-64-2; PO_4^{3-}, 14265-44-2.

References

Batzri, S., & Korn, E. D. (1973) *Biochim. Biophys. Acta 298*, 1015–1019.

Birrel, G. B., & Griffith, O. H. (1976) *Biochemistry 15*, 2925–2929.

Botin, H., & Mathis, P. (1985) *Biochemistry 24*, 6453–6460.
Brown, L. R., & Wüthrich, K. (1977a) *Biochim. Biophys. Acta 464*, 356–369.
Brown, L. R., & Wüthrich, K. (1977b) *Biochim. Biophys. Acta 468*, 389–410.
Chapman, D., & Urbina, J. (1971) *FEBS Lett. 12*, 169–172.
Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Ramshaw, J. A., Norris, V. A., & Venkatappa, M. P. (1978) *Nature (London) 272*, 319–324.
Cookson, D. J., Hayes, M. T., & Wright, P. E. (1980) *Biochim. Biophys. Acta 591*, 162–176.
Cusanovich, M. A., & Miller, W. G. (1974) *Bioelectrochem. Bioenerg. 1*, 448–458.
Fang, Y., & Tollin, G. (1983) *Photochem. Photobiol. 38*, 429–439.
Farver, O., & Pecht, I. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4190–4193.
Farver, O., Shahak, Y., & Pecht, I. (1982) *Biochemistry 21*, 1885–1890.
Freeman, H. C. (1981) *Coord. Chem. 21*, 29–51.
Green, D. E., & Fleischer, S. (1963) *Biochim. Biophys. Acta 70*, 554–584.
Gulik-Krzywick, T., Schechter, E., Luzatti, V., & Faure, M. (1969) *Nature (London) 223*, 1116–1121.
Gupte, S. S., & Hackenbrock, C. R. (1988a) *J. Biol. Chem. 263*, 5241–5247.
Gupte, S. S., & Hackenbrock, C. R. (1988b) *J. Biol. Chem. 263*, 5248–5253.
Guss, J. M., & Freeman, H. C. (1983) *J. Mol. Biol. 169*, 521–563.
Haehnel, W., Berzbron, R. J., & Anderson, B. (1981) *Biochim. Biophys. Acta 582*, 482–490.
Hauska, G., Berzbron, R. J., McCarty, R. E., & Racker, E. (1971) *J. Biol. Chem. 246*, 3524–3531.
Hurley, J. K., Castelli, F., & Tollin, G. (1980) *Photochem. Photobiol. 32*, 79–86.
Hurley, J. K., Castelli, F., & Tollin, G. (1981) *Photochem. Photobiol. 34*, 623–631.
Kimelberg, H. K., & Lee, C. P. (1969) *Biochem. Biophys. Res. Commun. 34*, 784–790.
Kimelberg, H. K., & Papahadjopoulos, D. (1971) *J. Biol. Chem. 246*, 1142–1148.
Koppenol, W. H., & Margoliash, E. (1982) *J. Biol. Chem. 257*, 4426–4437.
Lappin, A. G., Segal, M. G., Weatherburn, D. C., & Sykes, A. G. (1979) *J. Am. Chem. Soc. 101*, 2297–2301.
Meyer, T. E., Przysiecki, C. T., Watkins, J. A., Bhattacharyya, A., Simondsen, R. P., Cusanovich, M. A., & Tollin, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6740, 6744.
Mustonen, P., Virtanen, J. A., Somerharju, P. J., & Kinnuen, P. K. J. (1987) *Biochemistry 26*, 2991–2997.
Nicholls, P. (1974) *Biochim. Biophys. Acta 346*, 261–310.
Nicholls, P., & Malviya, A. N. (1973) *Trans. Biochem. Soc. 1*, 372–375.
Olsen, L. F. (1982) *Biochim. Biophys. Acta 582*, 482–490.
Overfield, R. E., & Wraight, C. A. (1980a) *Biochemistry 19*, 3322–3327.
Overfield, R. E., & Wraight, C. A. (1980b) *Biochemistry 19*, 3328–3334.
Papahadjopoulos, D., Moscarello, M., Eylar, E. H., & Isac, T. (1975) *Biochim. Biophys. Acta 401*, 317–335.
Quinn, P. J., & Dawson, R. M. C. (1969) *Biochem. J. 115*, 65–75.
Rich, P. R., Heathcote, P., & Moss, D. A. (1987) *Biochim. Biophys. Acta 892*, 138–151.
Senthilathipan, V., & Tollin, G. (1985) *Photochem. Photobiol. 42*, 437–445.
Senthilathipan, V., & Tollin, G. (1986) *Photochem. Photobiol. 43*, 545–550.
Senthilathipan, V., & Tollin, G. (1987) *Photochem. Photobiol. 46*, 537–542.
Simondsen, R. P., & Tollin, G. (1983) *Biochemistry 22*, 3008–3016.
Staudenmayer, N., Smith, M. B., Smith, H. T., Spies, F. K., Jr., & Millet, F. (1976) *Biochemistry 15*, 3198–3205.
Staudenmayer, N., Ng, S., Smith, M. B., & Millett, F. (1977) *Biochemistry 16*, 600–604.
Steinemann, A., & Laugher, P. (1971) *J. Membr. Biol. 4*, 74–86.
Stellwagen, E. (1978) *Nature (London) 275*, 73–74.
Swanson, R., Trus, B. L., Mandel, N., Mandel, G., Kallai, O., & Dickerson, R. E. (1977) *J. Biol. Chem. 252*, 759–775.
Szebeni, J., & Tollin, G. (1988) *Biochim. Biophys. Acta 932*, 153–159.
Takabe, T., Niwa, S., & Ishikawa, H. (1980) *J. Biochem. (Tokyo) 87*, 1335–1339.
Takanao, M., Takahashi, M., & Asad, K. (1982) *Arch. Biochem. Biophys. 218*, 369–375.
Takabe, T., Ishikawa, H., Niwa, S., & Tanaka, Y. (1984) *J. Biochem. (Tokyo) 96*, 385–393.
Tollin, G., Castelli, F., Cheddar, G., & Rizzuto, F. (1979) *Photochem. Photobiol. 29*, 147–152.
Tollin, G., Meyer, T. E., & Cusanovich, M. A. (1986) *Biochim. Biophys. Acta. 853*, 29–41.
Van, S. P., & Griffith, O. H. (1975) *J. Membr. Biol. 20*, 155–170.
Vanderkooi, J. M. (1976) *Biochem. Biophys. Res. Commun. 69*, 1043–1049.
Vanderkooi, J., Erecinska, M., & Chance, B. (1973a) *Arch. Biochem. Biophys. 154*, 219–229.
Vanderkooi, J., Erecinska, M., & Chance, B. (1973b) *Arch. Biochem. Biophys. 157*, 531–540.
Wainwright, P., Power, D. M., Thomas, E. W., & Davies, J. V. (1978) *Int. J. Radiat. Biol. 33*, 151–162.
Weber, C. P., & Tollin, G. (1985) *J. Biol. Chem. 260*, 5568–5573.
Yocum, C. F. (1982) in *Methods in Chloroplast Molecular Biology* (Edelman, M., Hallick, R. B., & Chua, N. H., Eds.) pp 973–981, Elsevier Biomedical Press, Amsterdam, The Netherlands.